

The Case for Laboratory Developed Procedures: Quality and Positive Impact on Patient Care

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Abstract

An explosion of knowledge and technology is revolutionizing medicine and patient care. Novel testing must be brought to the clinic with safety and accuracy, but also in a timely and cost-effective manner, so that patients can benefit and laboratories can offer testing consistent with current guidelines. Under the oversight provided by the Clinical Laboratory Improvement Amendments, laboratories have been able to develop and optimize laboratory procedures for use in-house. Quality improvement programs, interlaboratory comparisons, and the ability of laboratories to adjust assays as needed to improve results, utilize new sample types, or incorporate new mutations, information, or technologies are positive aspects of Clinical Laboratory Improvement Amendments oversight of laboratory-developed procedures. Laboratories have a long history of successful service to patients operating under Clinical Laboratory Improvement Amendments. A series of detailed clinical examples illustrating the quality and positive impact of laboratory-developed procedures on patient care is provided. These examples also demonstrate how Clinical Laboratory Improvement Amendments oversight ensures accurate, reliable, and reproducible testing in clinical laboratories.

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The field of pathology offers the opportunity to better understand the science behind the mechanisms of disease, to lead innovation of new diagnostic technologies, to provide quality oversight of these developments, and to have enormous impact on the lives of patients every day. Patients benefit from laboratory medicine testing throughout their lives as every medical decision can be impacted by the result of a laboratory test. Laboratory results constitute the majority of data in a patient's electronic medical record, and our procedures dictate the majority of downstream medical decisions for patients.^{1,2} Clinical laboratory medical professionals have the unique responsibility to patients for assessing the performance of technologies in providing the most accurate information to ensure the most appropriate and efficient course of care.

It is an understatement to say that the medical field is rapidly changing. Technology and new genomic data developed as a result of the human genome project are leading to an explosion of knowledge applicable to individual patient care; this is the promise of precision medicine. We must continue to innovate and integrate novel diagnostic tools, genomic information, and new treatments into clinical practice. Considerable technologic advances allow clinical laboratory professionals to offer new testing that provides more information than ever before and often within a time frame that allows rapid patient care and better outcomes. Next-generation sequencing (NGS) in genetics and oncology, MALDI-TOF in the clinical microbiology laboratory, and a variety of mass spectroscopy-based methods in clinical chemistry now allow precise and rapid testing with demonstrated improvements in patient care. It is often thought that when "laboratory tests" are done to reach a diagnosis, they are done with a kit or on a machine, but in fact, most are procedures done with the direct involvement of a laboratory professional or physician. Laboratory tests are generally not fully encompassed by a "test kit" but often start with the pathologist examining the tissue section, bone marrow aspirate, or gram stain and determining what additional information is needed to provide the clinician with the best scenario so that the patient can be treated most effectively. Often, clinical laboratory professionals lead the development and optimization of these approaches to improve care and fill a clinical need, and their involvement in the process helps ensure that the highest quality standards are maintained. Ongoing development of these novel methods is critical to medicine and must be done with the highest level of safety and accuracy, yet simultaneously addressing growing demands to lower the cost of medical care in the United States.

The regulatory oversight of laboratory-developed testing procedures (LDPs) has a critical impact on patients' access to

testing and diagnosis. Currently, there is national discussion regarding the optimal regulatory oversight of laboratory tests and procedures that will balance the needed accuracy and safety with ensuring that new tests are made available to patients safely and expeditiously. Oversight provided by the Clinical Laboratory Improvement Amendments (CLIA) and the Food and Drug Administration (FDA) currently exists in the clinical laboratory. It must be noted that a spectrum of testing activities take place in clinical laboratories. These activities range from complex procedures, such as evaluation of a tissue biopsy, classification of a tumor, or culture of a microbe, to more automated or standardized tests for which an FDA-approved kit is utilized. All of these activities take place under the direction of a laboratory professional and in accordance with the detailed requirements of CLIA.

The FDA review process is well suited for diagnostic assays that are commercially marketed as kits designed to operate across a spectrum of laboratory settings, in laboratories with a range of expertise. Currently, FDA approval or clearance requires prospective clinical trial data and a lengthy review process, and thus, FDA approval takes considerable effort and time. The investment needed for this process impacts the types of tests, and also sample types, that are submitted for approval, as manufacturers must recover their investment afterward. Additionally, FDA approval for an in vitro diagnostic (IVD) specifies the sample type, clinical purpose, and other aspects of performance of the assay. When a new clinical need for the IVD arises, the need to use a new sample type arises, or any needed modification that arises in response to the ongoing and sometimes rapid advancements seen in medicine, incorporation of these improvements renders the test an LDP, with the validation needs of an LDP under CLIA. Laboratories can be caught between the regulations and the needs to best serve their patients.

The CLIA provide for oversight of clinical laboratories by defining all aspects of laboratory operation, including the quality programs required for clinical tests, personnel requirements, and the validation requirements for LDPs. The CLIA certification of laboratories can be accomplished through several deemed agencies such as the College of American Pathologists (CAP) or the New York State Department of Health (NYSDOH). These organizations provide operational guidelines and perform on-site inspections based upon checklists developed via consensus of laboratory experts, which include hundreds of pages of requirements and data points. Compliance with CLIA has been built into clinical laboratory operations, mechanisms for data collection, training, proficiency testing (PT), and test implementation. Laboratories are subject to unannounced inspections and must demonstrate satisfactory performance

characteristics for any test offered to ensure that results are accurate. For testing not reviewed by the FDA, such as LDPs, laboratories must go through a rigorous validation process before offering the test for clinical use. Under CLIA, the validation data collected by these laboratories are subject to ongoing peer review. In particular, organizations with deemed status overseeing laboratories (such as CAP and NYSDOH) conduct rigorous peer-inspection using detailed criteria developed specifically for molecular pathology. Laboratories also participate in required PT to demonstrate assay quality, with interlaboratory data sharing and assessment (most often led by the CAP) that leads to ongoing broad improvement in LDPs.

Recent public documents have presented examples of specific LDPs in which the clinical validity of the test, the interpretation or use by clinicians, or the reproducibility of the laboratory data was questioned, raising concern about the safety and efficacy of this category of tests.^{3,4} Enhanced regulatory oversight has been proposed to help ensure laboratories are delivering meaningful, high-quality results to patients. However, additional regulations have the potential to slow innovation and to limit and delay patient access to novel testing that impacts their clinical care, as well as add redundant reporting efforts and significant cost. Expanded oversight of laboratories through a revised CLIA process has also been proposed (<http://www.cap.org/ShowProperty?nodePath=/UCMCon/Contribution%20Folders/WebContent/pdf/2015-cap-ldt-legislative-proposal.pdf>. Accessed April 27, 2017).⁵ In an effort to illustrate the need for and positive impact of laboratory-developed procedures on patient care, the following series of vignettes have been assembled. In each case, timely and high-quality laboratory-developed procedures filled a key clinical need. The impact on patient care has been summarized. As possible, data describing interlaboratory comparisons are provided to demonstrate the quality of these LDPs, as validated and performed in accordance with CLIA. These examples also serve to highlight the quality efforts and interlaboratory comparisons that take place before LDPs are offered by laboratories for clinical use; these activities and data are often unknown to the clinicians who utilize these testing services. Table 1 provides a guide to the examples included and summarizes the impact of each assay along with key points about its utility.

Clinical Analytes: Microbes

Herpes Simplex Virus Detection by Polymerase Chain Reaction

Encephalitis is the most serious complication of herpes simplex virus (HSV) infection.⁶ While rare, HSV is commonly included among causes of viral encephalitis. Since HSV infection can be treated effectively with acyclovir, it is critical to rapidly and accurately establish the diagnosis and initiate treatment to reduce morbidity and mortality. Culture of cerebrospinal fluid (CSF) is insensitive for the diagnosis of HSV encephalitis, so brain biopsy, a very invasive procedure with significant morbidity, was historically required to make a diagnosis, with

results not available for days afterward. Several studies demonstrated that detection of viral DNA by polymerase chain reaction (PCR) using CSF samples performed equivalently to brain biopsy, including a landmark study in 1995.⁷⁻⁹ Given the ease of collecting CSF samples, the lower risk of complication, the lower cost, and speed of results, PCR became the standard of care for diagnosing HSV CNS infections in the mid-1990s, with LDPs used for nearly 20 years. It was not until 2014 that the first FDA-cleared PCR test for the diagnosis of HSV encephalitis became available, with a second test cleared in 2015. Laboratory-developed testing procedures dramatically improved the quality of care for many patients and continue to be used successfully today, as the 2 cleared tests have limitations, requiring specific instrumentation not standard in many laboratories. Additionally, one of the tests is highly multiplexed, which may not be appropriate in all clinical situations.

Herpes simplex virus can also cause infections in neonates, with a frequency of 1:3500 to 1:5000 deliveries in the United States. The infection is acquired by exposure to maternal genital secretions. The presentation of neonatal HSV varies and includes skin, eye, and mouth infection, with encephalitis and disseminated disease in over 50% of cases. Again, rapid diagnosis is needed to prevent morbidity and sequelae from encephalitis. The diagnosis of encephalitis is made via PCR of CSF samples, while testing of plasma or serum is critical to diagnose disseminated disease. The ability to use plasma or serum is very important as it may be difficult to obtain enough CSF from a newborn to assess all diagnoses in the differential. Although there are 2 FDA-cleared assays to test CSF for HSV, there are no assays cleared for testing serum and plasma. Laboratory-developed testing procedures continue to play a critical role in the diagnosis and management of disseminated HSV infection in newborns.

Additionally, LDPs have performed with a high degree of accuracy and interlaboratory agreement. Blinded PT samples were distributed to 383 laboratories as part of a CAP PT survey for analysis of HSV; 91.6% correctly identified HSV-2, and 7.8% identified HSV, but not noting the subtype; an overall accuracy of 99.4% was obtained. Previous proficiency surveys showed similar results for samples containing HSV1. Over 90% of the laboratories used LDPs.¹⁰

BK Virus Detection by PCR

BK virus infection is very common, with a seroprevalence in the adult population of ~90%. After primary infection, the virus colonizes the renal and urinary tracts; healthy individuals will occasionally shed virus in their urine without consequence. However, in renal transplant recipients, BK virus is the major cause of polyomavirus-associated nephropathy (PVAN), putting 1% to 15% of kidney transplant patients at risk of premature allograft failure.¹¹ Given the lack of effective antiviral therapy for BK virus, the key to preventing allograft loss is to identify at-risk renal transplant recipients early and reduce immunosuppressive therapy before PVAN develops. Reduction of immunosuppressive therapy helps control viral replication and in most cases prevents the development of PVAN.¹²

Table 1. Examples of LDPs and Key Point Summary.

Analyte	Impact				Key Point
	Improved Diagnostic Performance	Faster Diagnosis	Cost Savings	Provided Data for Later IVD Kit	
Microbes					
HSV PCR	X	X	X	X	Avoid brain biopsy
BK PCR	X	X	X	Not yet	Improved management/outcome of renal transplant patients
CMV qPCR	X	X	X	X	Improved management/outcome of solid organ transplant patients
HPV in oropharyngeal cancer	X				Aids in treatment choice, prognostic information
PCR for emerging pathogens	X	X		X	Rapid diagnosis, may limit outbreak, public health advantages
Cancer					
KRAS mutations	X		X (avoids ineffective treatment)	X	Facilitates appropriate treatment choices
BRAF mutations	X		X (avoids ineffective treatment)	X	Facilitates appropriate treatment choices
MSI	X				Defines MSI-H and Lynch syndrome, facilitates appropriate treatment and screening
EGFR mutations	X		X (avoids ineffective treatment)	X	Facilitates appropriate treatment choices
NGS	X		X (compared to single-gene testing)		Facilitates appropriate treatment choices
BCR-ABL qPCR	X			X	Fundamental to CML patient management
Genetics					
Fragile X	X	X		X	Only means to positive diagnosis and carrier testing
Heritable gene panels by NGS			X (compared to single-gene testing)		
Cardiomyopathy	X	X			Only means for definitive diagnosis, appropriate treatment
Epilepsy	X	X			Definitive diagnosis, appropriate treatment
Neuromuscular disorders	X	X			Definitive diagnosis, appropriate treatment
Heritable Cancer Panels					Appropriate cancer screening
Whole exome sequencing	X				Definitive diagnosis of complex diseases
Huntington disease		X			Only means for presymptomatic disease diagnosis
Other					
Busulfan	X				Therapeutic drug monitoring, needed to prevent graft failure and neurotoxicity
Testosterone	X				Standardized and accurate method
Ethylene glycol		X			Only method to detect/monitor poisoning
Thyroglobulin	X				More accurate than FDA IVDs; improved care after thyroidectomy
Antimicrobial susceptibility	X				More accurate and current assessment of antimicrobial resistance than outdated FDA-approved breakpoints

Abbreviations: CML, chronic myelogenous leukemia; CMV, cytomegalovirus; EGFR, epidermal growth factor receptor; FDA, Food and Drug Administration; HPV, human papilloma virus; HSV, herpes simplex virus; IVD, in vitro diagnostic; NGS, next-generation sequencing; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction.

There is a critical balance between too much immunosuppressive therapy, which can lead to PVAN, and too little immunosuppression causing rejection. The ability to monitor BK virus levels in the blood allows for informed clinical decisions. Studies have shown that monitoring patients with viral load testing during the first 2 years after transplant can dramatically reduce the development of PVAN. Consensus guidelines recommend that screening for BK replication be performed at least every 3 months during the first 2 years posttransplant and then annually until the fifth year posttransplant.¹³ When the plasma viral load value rises above a threshold (10 000 to 50 000 copies/mL), a renal biopsy may be performed to assess for PVAN, and immunosuppressive therapy is reduced based on the results of the biopsy. Viral load testing is also done if there is an increase in serum creatinine, as the level of BK virus aids in distinguishing rejection from PVAN.

Although BK viral load testing has been the standard of care for several years and is used in transplant centers across the country, there is no FDA-cleared or FDA-approved test available. All testing is performed using LDPs. If these LDPs were not available, most cases of PVAN would not be identified promptly, leading to negative patient outcomes such as allograft loss or rejection.

Quantitative Cytomegalovirus Detection by PCR

Cytomegalovirus (CMV) can cause a wide range of complications among both solid organ transplant and hematopoietic stem cell transplant recipients, as well as in other immunocompromised patients. Cytomegalovirus has a high seroprevalence, and large numbers of transplant patients experience reactivation or primary infection, with probability increasing based on pre-transplant serostatus, severity of pretransplant conditioning, and allograft relatedness.^{14,15} Although infection can be subclinical, high or increasing viral load signals increased the risk of symptomatic disease, which can range from relatively mild constitutional symptoms to severe end-organ infection or potentially fatal disseminated disease. Preemptive therapy of high-risk patients based on the detection of increasing CMV load in peripheral blood was shown to be effective in the 1990s^{16,17}; however, the first FDA-approved IVD test did not appear on the market until 2012.¹⁸ In the intervening years, laboratory-developed assays played a critical role in bridging the gap. The presence of such methods and their adoption across the country enabled the routine use of CMV screening for asymptomatic patients in transplant centers soon after data supported its utility. The availability of such methods has likely saved many lives over the years, supporting early diagnosis, preemptive treatment strategies, and the assessment of therapeutic treatment efficacy.^{14,18-20} The widespread use of LDP CMV quantitative methods produced a generation of transplant physicians comfortable with the use of such methods and changed the epidemiology of posttransplant CMV disease, markedly reducing the incidence of early disease in such patients. Over the years of LDP use, numerous studies focused on continuous improvement and optimization of methods,²¹ including

the development of international quantitative standards in 2010.²² The ability to rapidly incorporate advances in technology (including the advent of real-time quantitative methods) has been demonstrated by the improvement in sensitivity and other performance characteristics of such tests over time. The data accumulated throughout these experiences informed the development of the first commercially available CMV IVD assays. In fact, the absence of LDPs and the vast clinical and laboratory experience that they provided likely would have delayed the availability of commercial methods, and their performance characteristics taken longer to optimize to today's levels.

Human Papilloma Virus in Oropharyngeal Cancers

Human papilloma virus (HPV), the causative agent of genital warts, has been implicated in the development of ~99% of cervical cancers.²³ More than 200 HPV genotypes have been described,²⁴ and approximately 40 are capable of infecting the human genital tract.²⁵ Of these, a relative few are known to cause cervical cancer and other malignancies; these can be identified by genotyping. Today, 4 assays are FDA approved for detecting high-risk HPV genotype strains in cervical specimens.²⁶⁻²⁹ These tests are routinely used to confirm the presence of an HPV infection, screen for cervical cancer, and refer cases with an indeterminate cytology examination.³⁰

During the past decade, a rise in oropharyngeal squamous cell cancer, primarily in 40 to 55-year-old Caucasian males with limited alcohol and tobacco exposure, has been described.³¹ Unexpectedly, investigators discovered the frequent presence of high-risk HPV genotype strains in these lesions. Human papilloma virus–positive head and neck cancer is biologically distinct from HPV-negative disease. Patients with HPV-driven tumors have a significantly better prognosis, including response to chemoradiation therapy and overall survival, compared to HPV-negative patients.³² Therefore, testing head and neck cancer specimens for high-risk HPV genotypes has become standard of care and is used to guide treatment.^{33,34} However, the HPV tests that are FDA approved for cervical specimens have not been approved for head and neck cancer, leaving a critical gap for patient care. Clinical laboratories have thus had to develop new assays or modified the existing FDA-approved ones to detect high-risk HPV genotypes in head and neck cancer specimens. Inclusion of patients in several clinical trials is based on these LDPs (www.clinicaltrials.gov. Accessed April 27, 2017). Without laboratory-developed tests, patients with head and neck cancer cannot benefit from personalized treatment strategies.

Detection of Emerging Infectious Diseases

Clinical laboratories must be highly vigilant for infectious disease outbreaks, since they are likely the first to recover the pathogen and recognize the potential for an outbreak. Recent examples of emerging or reemerging pathogens causing substantial human morbidity and mortality include avian influenza virus ("bird flu"), chikungunya virus, Ebola virus, Middle Eastern respiratory syndrome virus, severe acute respiratory

syndrome virus, and Zika virus.^{35,36} At the time of their emergence, no FDA-approved tests were available to detect any of these pathogens. In response, many clinical laboratories developed, validated, and implemented the LDPs needed to care for patients at their institutions.^{37,38} These assays were based on extensive data sets and reported in peer-reviewed journals, supporting their quality claims. Since performance characteristics such as analytic sensitivity (limit of detection) may vary between different assay designs,³⁹ as was recently discussed for several different Zika virus tests,⁴⁰ these sorts of collaborative efforts are essential. In many cases, laboratories leading these efforts collaborated with one another to share validation materials, perform interlaboratory comparisons, and exchange blinded testing samples (<http://www.usatoday.com/story/news/2016/02/23/texas-hospitals-develop-rapid-zika-test/80776382/>. Accessed April 27, 2017). While perhaps not ideal, there is an urgent need for a more integrated and coordinated mechanism for rapid diagnosis of novel infectious agents that incorporates both the public health and hospital laboratories.

The recent emergence of Zika virus, which has been linked to severe birth defects,⁴¹ underscores the need for clinical laboratories to have rapid access to diagnostic tools. When the Secretary of Health and Human Services declared Zika virus to be a public health emergency, the first FDA Emergency Use Authorization for Zika virus testing was granted to a test available only to the public health laboratory system, not to hospital laboratories on the front lines of patient care (<https://www.federalregister.gov/documents/2016/03/28/2016-06888/authorization-of-emergency-use-of-an-in-vitro-diagnostic-device-for-diagnosis-of-zika-virus>. Accessed April 27, 2017). As a result, the public health system quickly became overwhelmed, as it has been during past outbreaks, such as influenza.⁴² In many areas, turnaround times for Zika virus testing exceeded 4 to 8 weeks (http://www.nytimes.com/2016/09/13/us/zika-test-delays-florida-pregnant.html?_r=0. Accessed April 27, 2017, <http://medicalxpress.com/news/2016-10-lab-constraints-zika-results.html>. Accessed April 27, 2017, <http://www.miamiherald.com/news/health-care/article104856631.html>. Accessed April 27, 2017). These delays critically impact patient care, particularly for pregnant women with possibly affected fetuses. In comparison, many hospital-based and national reference laboratories are able to report results within hours (<http://www.usatoday.com/story/news/2016/02/23/texas-hospitals-develop-rapid-zika-test/80776382/>. Accessed April 27, 2017, http://www.nytimes.com/2016/09/13/us/zika-test-delays-florida-pregnant.html?_r=0. Accessed April 27, 2017). For example, in Miami-Dade Florida, where more than 1000 people have been confirmed Zika positive and the virus is circulating in the local mosquito population, the public health laboratory system encountered a backlog of nearly 1000 untested specimens (<http://www.miamiherald.com/news/health-care/article104856631.html>. Accessed April 27, 2017). Diagnostic assays for Zika and other emerging pathogens will continue to evolve,⁴³ but it is clear that rapid identification of pathogens during other outbreaks facilitates rapid treatment and appropriate isolation of patients, leading to improved patient outcomes

and potentially slowing the spread of infections such as influenza (<http://www.cdc.gov/flu/professionals/diagnosis/molecular-assays.htm>. Accessed April 27, 2017). Implementation of the first rapid diagnostic tests for influenza was directly associated with reduced length of hospital stay, decreased mortality, and reduced costs.⁴⁴ Access to diagnostic tests, early in the course of an outbreak and in hospital laboratories, has a positive public health impact.

Cancer Targets

Mutation Detection in KRAS and RAS Family Genes

The *KRAS* gene encodes a GTPase critical in signal transduction that is known to be mutated in a wide range of tumor types.⁴⁵ A landmark study presented at the American Society of Clinical Oncology (ASCO) meeting in 2007 demonstrated that patients with metastatic colorectal cancer harboring a mutated *KRAS* failed to respond to targeted therapy with cetuximab.^{46,47} At the time, there were no clinical tests for *KRAS* mutations available. Molecular pathology laboratories worked quickly to fill this need, to define the best analytic approaches, and to ensure that test results done in one laboratory matched those done in another.⁴⁸⁻⁵⁰ Within a few months, laboratories were able to offer fully validated *KRAS* assays that worked reliably and were safe for patient care. Under CLIA, the validation data collected by these laboratories were subject to ongoing peer review, and laboratories participate in ongoing PT to demonstrate consistent assay quality.

In 2009, the ASCO and the National Comprehensive Cancer Network (NCCN) recommended mutational profiling of *KRAS* exons 12 and 13 before institution of anti-epidermal growth factor receptor (EGFR) therapy for patients with metastatic colorectal cancer; it became standard of care to assess formalin-fixed paraffin-embedded tumor tissues from patients with metastatic colon cancer for *KRAS* mutation status.^{51,52} It was not until 5 years later that the FDA cleared QIAGEN's therascreen *KRAS* test, designed to detect the presence of 7 mutations in the *KRAS* gene in colorectal cancer.⁵³ By this time, new data demonstrated that *KRAS* analysis alone was not enough; mutation analysis of other genes was necessary,^{54,55} and the FDA-approved assay was already inadequate for patient testing compliant with national treatment guidelines. Without LDPs, an estimated 10% of patients with non-exon 2 *KRAS* mutations would be overtreated with expensive anti-EGFR therapy.⁵⁶ The use of LDPs has thus persisted in clinical practice to provide patients with the most complete information to guide treatment.

In the CAP *KRAS*-B-2015 mailing, 204 laboratories reported results from testing 3 blinded proficiency-testing specimens. The specimens contained recurring somatic mutations in *KRAS* exons 12 or 13 (NM_004985.3), c.35G>T (p.G12V), and c.38G>A (p.G13D). An acceptable response was reported by over 96% of the laboratories for both mutations (197/204 for p.G12V and 195/202 for p.G13D). The vast majority of reporting laboratories utilized LDPs.⁵⁷

KRAS and *RAS* family gene mutation analysis is also critical in the management of patients with non-small-cell lung cancer (NSCLC) and other tumors,⁵⁸ for which FDA approval of kits has not occurred; LDPs or off-label use of kits is required.

***BRAF* Mutation Detection**

BRAF belongs to a family of serine–threonine protein kinases that participate in signal transduction cascades involving *RAS*, *RAF* MEK, and *ERK* family members. This pathway is important in the regulation of normal cell proliferation and differentiation.⁵⁹ Activating mutations in *BRAF* can lead to increased proliferation and prolonged cell survival in a variety of tumor types.⁶⁰ Laboratories will typically test for *BRAF* mutations in low-grade gliomas (for diagnosis), colorectal cancer (to establish the sporadic origin of MSI-H tumors and response to anti-EGFR therapy), hairy cell leukemia (for diagnosis), lung adenocarcinomas (to predict response to therapy), thyroid cancer (for preoperative detection of thyroid cancer in FNA samples and prognosis in papillary thyroid carcinoma), or melanoma (to predict response to *BRAF* kinase inhibitors).

For one of these indications, malignant melanoma, FDA-approved companion diagnostics are available. Vemurafenib (ZELBORAF) is a kinase inhibitor indicated for the treatment of patients with unresectable or metastatic melanoma with *BRAF* V600E mutation. Dabrafenib (TAFINLAR) is a kinase inhibitor indicated as a single agent for the treatment of patients with unresectable or metastatic melanoma with *BRAF* V600E mutation or in combination with trametinib (MEKINIST) for the treatment of patients with unresectable or metastatic melanoma with *BRAF* V600E or V600K mutations (www.fda.gov. Accessed April 27, 2017).

The Cobas 4800 *BRAF* V600 Mutation Test (Roche Molecular Systems, Pleasanton, CA, USA) sporadically cross reacts with *BRAF* V600K and *BRAF* V600D mutations^{61,62} and thus is neither sensitive for *BRAF* V600 mutations nor specific for *BRAF* V600E mutations, confounding accurate outcome evaluations and preventing its usefulness in selecting patient for Tafenlar therapy. The THxID *BRAF* kit (bioMerieux, Boston, MA, USA) does detect both *BRAF* V600E and *BRAF* V600K mutations (but not other *BRAF* V600 mutations) and is necessary to distinguish between alternate therapeutic options (single agent vs combination therapy). It is important to note that increased cell proliferation has been seen in tumors treated with *BRAF* inhibitors with normal *BRAF*.⁶¹ It is therefore important to identify all *BRAF* activating mutations to assist in the selection of appropriate therapy. The FDA-approved assays are therefore not adequate for current clinical needs.

In a 2015 European multicenter study,⁶² 420 consecutive tumor samples of histologically proven melanoma tumor tissue were assessed for *BRAF* mutation status by the Cobas system and a variety of laboratory developed procedures. Testing was concordant for 392 (93.3%) of 420 samples but discordant for 28 (6.7%). Among the discordant cases, 11 had invalid results (8 samples with the Cobas and 3 with LDPs). Of 10 samples with *BRAF* V600 mutations detected by the

LDPs (but not by the Cobas Mutation Test), 5 were V600K, D, or R mutations, and 2 contained only 20% tumor cells. For the 7 samples with *BRAF* V600 mutations detected by the Cobas but not by the LDPs, 4 were confirmed with retesting, 1 was not mutated, and 2 were considered invalid results. This study documents similar results between the performance of *BRAF* LDPs and IVDs.

Further data can be gathered from the CAP PT program. In the *BRAF*-B-2015 CAP proficiency survey, 173 laboratories reported on the detection of V600E and other *BRAF* mutations by a variety of analytic methods. Two of the well-characterized specimens in the proficiency test contained *BRAF* V600E alleles, and 98.8% and 99.4% of laboratories correctly reported the mutation. The majority of these laboratories used LDPs.⁶³

There are no currently available *BRAF* mutation tests approved for use in other tumor types such as those mentioned above, and use of the existing IVD tests would constitute off-label use and hence LDPs.

Microsatellite Instability

Microsatellite instability is the presence of hypermutability in repetitive DNA sequences resulting from impaired DNA mismatch repair. Microsatellite instability can be an inherited or acquired feature of tumors. Microsatellite instability occurs in approximately 15% of all colorectal carcinomas and is a consistent feature of colorectal and other tumors in patients with Lynch syndrome.⁶⁴ Tumors are classified as showing high levels of MSI (MSI-H phenotype) if 2 or more of 5 microsatellite markers (or $\geq 30\%$) exhibit instability, a microsatellite-stable phenotype if none of the markers show instability, and an MSI-low phenotype if only 1 of 5 or less than 30% of the markers show instability.⁶⁵ Studies have confirmed that the appropriate cutoff for determining an MSI-H phenotype is the finding of instability in 30% or more of the markers tested. The finding of an MSI-H phenotype is consistent with the presence of defective DNA MMR in the tumor.⁶⁶

The finding of an MSI-H phenotype in a CRC increases the likelihood that the patient has LS but is not specific for LS. The definitive establishment of a diagnosis of LS requires the finding of a pathogenic germline mutation in one of the DNA MMR genes. Additional testing that can be offered to determine whether a patient with an MSI-H CRC is likely to have LS includes testing the tumor for DNA MMR protein expression using IHC, *BRAF* V600E point mutation analysis (since *BRAF*-mutated MSI-H colorectal carcinomas are known to have sporadic MMR gene mutations), and MLH1 promoter hypermethylation.

Studies have shown that an MSI-H phenotype is a favorable independent prognostic indicator in patients with CRC.⁶⁷ In addition, some reports indicate that MSI-H tumors may not be responsive to 5-fluorouracil-based therapies.⁶⁸ Recent draft guidelines developed collaboratively by 4 professional societies recommend that deficient mismatch repair/microsatellite instability testing must be performed in all colorectal cancers for prognostic stratification and identification of patients with Lynch syndrome.⁶⁹ Although numerous laboratories offer MSI

testing using LDPs, there are currently no FDA-approved tests for the evaluation of microsatellite instability. A summary of CAP PT results⁷⁰ demonstrates excellent performance of laboratories participating in the MSI proficiency surveys. This good performance of laboratories over the years may be partly due to the educational nature of the CAP PT, which provides laboratories with an external mechanism to monitor the quality status of their testing.

Epidermal Growth Factor Receptor Mutation Detection

Epidermal growth factor receptor is a membrane-bound tyrosine kinase which activates several signaling pathways known to be altered in human cancer, including NSCLCs.⁷¹⁻⁷³ Non-small cell lung cancer tumors with EGFR-activating mutations are responsive to gefitinib and erlotinib, small molecule tyrosine kinase inhibitors of EGFR.^{74,75} The FDA approval of anti-EGFR therapies based on clinical trial outcomes data resulted in the need for clinical laboratories to test tumor tissue for the EGFR-sensitizing mutations in order for patients to be eligible for treatment.

With no FDA-approved “companion” diagnostic test on the market, CLIA-licensed laboratories developed and validated LDP tests for the 2 most common *EGFR* mutations as early as 2004.⁷³ The FDA followed with approval of the Roche Cobas *EGFR* Mutation Test in 2013 along with the Qiagen therascreen *EGFR* RGQ Kit. Both assays tested for the exon 19 deletions and the exon 20 L858R point mutation. Of note was that each test was approved for specific therapeutic indications and specimen types. As new drugs became available, approval for new claims was needed. Laboratory-developed procedures continue to be the method of choice due to the limitations of claims made for FDA-approved assays and performance characteristics, including types of mutations being detected.⁷⁶

Clinical laboratories participate in twice-yearly proficiency test challenges of unknown samples that must be analyzed and reported, with results graded and compared to other laboratories performing the testing. In the CAP *EGFR*-B-2015 proficiency test, 192 laboratories reported results from testing 3 unknown proficiency-testing specimens in late 2015. The specimens contained 3 recurring somatic mutations in *EGFR* (NM_005228.3): c.2369C>T (p.T790M), c.2573T>G (p.L858R), and c.2582T>A (p.L861Q); these mutations were detected by 98.3% (178 of 181), 99.0% (190 of 192), and 94.1% (144 of 153) of laboratories. Note: Some laboratories do not test for certain mutations, hence, the denominator is often less than 192.⁷⁷

As patients being treated with these new targeted anti-EGFR therapies began to relapse, further studies revealed that *EGFR* harbors both sensitizing and resistance mutations. The CLIA laboratories have demonstrated the ability to detect all mutations in the *EGFR* gene as well as in other genes using NGS assays to sequence panels of cancer-related genes.⁷⁸ This panel approach allows the laboratory to provide the oncologist with a more comprehensive profile of the tumor, using a cost-effective technology that makes maximal use of small tissue samples and thus makes treatment strategies more effective. In

addition, the time saved by testing a broader panel of gene targets can result in better outcomes for the patient as well as fewer adverse drug reactions. For the payer, the cost savings of such an approach versus algorithm-based testing with single gene assays is significant. Currently, there are no FDA-approved sequencing assays for *EGFR* mutation status, and LDPs are solely used for the detection of these mutations that define therapy selection.

Next-Generation Sequencing

The complexity of cancer biology and the ever-evolving therapeutic approach to management of the patient with cancer more often requires expanded knowledge of the tumor beyond single-gene mutation status. Over the past several years, the laboratory's ability to multiplex testing for several genes or genetic variants has been limited by the available technologies. Next-generation sequencing or massively parallel sequencing has allowed the laboratory to provide a more comprehensive genomic profile of tumor cells in a single assay than previous methods. The ability to detect numerous mutations in multiple genes results in information that can allow the oncologist to develop a more accurate treatment strategy including therapies selected based on a “responsive” tumor profile and those not selected based on the presence of resistance mutations.⁷⁹⁻⁸¹

Instrumentation from Thermo Fisher (Thermo Fisher Life Technologies, Carlsbad, CA, USA) and Illumina (Illumina, San Diego, CA, USA), the Personal Genome Machine (PGM), and MiSeq, have made NGS suitable for routine clinical laboratory testing.⁸²⁻⁸⁴ In 2015, the MiSeq Dx obtained FDA approval. Despite this approval and the routine use of NGS in LDPs setting, there are no currently FDA-approved NGS tests for application in oncology. Although many drug package inserts require or allude to the use of a companion diagnostic for eligibility, very few companion diagnostic tests are FDA approved and none using NGS technology. As an LDP, clinical laboratories are required to demonstrate rigorous performance criteria for wet-bench testing and analysis pipelines to ensure the test is functioning properly for its intended clinical purpose.^{73,85-87}

Most NGS testing for therapeutic selection in cancer consists of panels of genes that range from 10 to 400 or more genes. Each test can be designed to detect hotspots of known mutations in those genes, to sequence the entire coding region of the genes, or to sequence the entire gene. The end result is a comprehensive profile of the tumor genome that can then be used to tailor therapy for the individual patient. Although NGS assays cost more than single-gene assays, the cost per gene sequence is dramatically reduced and results in cost savings over using multiple single-gene tests. Furthermore, NGS panels can be applied to very small specimen samples, using as little as 10 to 250 ng of input DNA depending on the analytic platform utilized. Since evaluation of therapeutic biomarkers is usually needed in the setting of advanced disease and such patients often have only limited tissue samples available for testing, NGS assays allow for much more extensive genomic information to be obtained

compared to single-gene assays, each of which can require DNA input comparable to that of an entire NGS panel.

A total of 111 laboratories recently participated in a CAP-sponsored proficiency assessment of NGS cancer panel testing (NGSST-A-2016), with data collection on 10 gene mutations (*AKT1*, *ALK*, *BRAF*, *EGFR*, *FBXW7*, *IDH1*, *KIT*, *KRAS*, *NRAS*, and *PIK3CA*). Of 1010 genotyping calls across the spectrum of mutations tests, 993 (98.3%) were called concordantly (unpublished data).⁸⁸

***BCR-ABL1* PCR for Monitoring Targeted Therapy in Chronic Myelogenous Leukemia**

No other LDP in the field of oncology has had a greater impact on patient care than has the quantitation of RNA in chronic myelogenous leukemia (CML). One of the first (and arguably most successful) molecularly targeted cancer therapies is the *BCR-ABL1*-targeted tyrosine kinase inhibitor, imatinib, which was FDA-approved in 2001. In those very early days of targeted therapy, long before the advent of FDA-approved “companion diagnostics,” the ubiquitous and obvious method to determine the efficacy of novel leukemia treatments was to directly quantitate the target of the inhibitor drug, namely, the cancer cell-specific *BCR-ABL1* fusion gene. A reduction in posttreatment *BCR-ABL1* RNA levels, as measured by sensitive laboratory-developed PCR-based methods, was shown to be the best available test for predicting therapeutic response and long-term progression-free survival in TKI-treated patients with CML.⁸⁹ Consensus oncology practice guidelines in both the United States (NCCN)⁹⁰ and Europe (ELN),⁹¹ going back at least a decade, have universally recommended that TKI-treated patients with CML should be serially monitored with a (laboratory-developed) *BCR-ABL1* RT-PCR blood test at least every 3 to 6 months during their lifelong course of TKI therapy. The NCCN and ELN guidelines have also long recommended serial *BCR-ABL1* RNA testing to directly inform not only the appropriate dose of TKI (to overcome developing resistance) but also the therapeutic switch from one TKI to another (depending on the drug’s known resistance profile).

To directly support optimal therapeutic decision-making in the routine care of patients with CML, clinical laboratories have been offering accurate and sensitive PCR-based laboratory-developed procedures for *BCR-ABL1* for at least the last 15 years. Recognizing that standardization of these LDP’s was necessary to promote uniform therapeutic decision-making, the laboratory community undertook an extensive multiyear project to create a standardized “international scale” (IS) of measurement for *BCR-ABL1* messenger RNA.⁹² Follow-up efforts resulted in the creation of a World Health Organization-recognized panel of reference materials directly linked to the *BCR-ABL1* IS⁹³ and the subsequent creation of secondary IS-calibrated reference materials that could be used for routine daily QC in clinical laboratories.^{94,95} Recognizing the additional need for PT, the CAP has been offering semi-annual *BCR-ABL1* PT surveys for at least 10 years, with a progressive increase in the number of participating laboratories

(from ~100 in 2009 to ~190 in 2016). As proof of the near-universal recognition of assay standardization, approximately 90% of these accredited laboratories now report their PCR results using the standardized IS. A 2016 CAP survey confirmed excellent interlaboratory precision, with over 90% of laboratories reporting a *BCR-ABL1* IS result within internationally acknowledged acceptable tolerance limits (0.5 logs) for IS reporting of a sample with an approximate 1000- to 10 000-fold reduction in pretreatment *BCR-ABL1* levels.⁹⁶

The primary driving force behind the remarkable increase in longevity and quality of life for patients with CML over the past 15 years has no doubt been the availability of novel-targeted TKI therapies. This has become the paradigm for personalized/precision cancer medicine programs, coupled with parallel effort of the laboratory community toward building, improving, and standardizing accurate, precise, and sensitive laboratory-developed tests for *BCR-ABL1*. Of note, this 15-year targeted therapy program for CML occurred entirely without the availability of FDA-approved *BCR-ABL* diagnostic reagents, which have only become available in 2016. These FDA-approved assays were based upon those developed in clinical laboratories; they are not approved for diagnosis of CML nor do they cover the spectrum of breakpoints that occur in the disease. During those ground-breaking first 15 years of the targeted cancer therapy era, if the laboratory community had been prohibited from providing high-quality, standardized LDP-based testing under existing CLIA guidelines, the negative consequences to patient care in the past and the future would have been substantial.

Genetic Tests

***Fragile X* Testing**

Fragile X (FX) syndrome is one of the most common inherited causes of intellectual disability. The causative molecular mechanism is an expansion of a CGG repeat region of the 5’ regulatory region of the *FMRP* gene. When the CGG repeat expands beyond approximately 200 CGG repeats, the gene is methylated and silenced. Laboratory testing for FX includes sizing the number of repeats as well as methylation analysis and has been available for clinical diagnostic and carrier status testing for over 20 years. The American College of Medical Genetics and Genomics (ACMG) has published practice guidelines for appropriate test ordering.⁹⁷ It is a first tier test for individuals and families in which an X-linked inheritance pattern of intellectual disability is suspected. Once an expanded FX allele has been identified, other family members can be tested to identify premutation carriers at risk of having affected offspring. Prenatal (fetal) or preimplantation genetic diagnostic testing is available for known FX carriers.

According to the Genetic Test Registry, over 50 laboratories in the United States offer testing for FX (<https://www.ncbi.nlm.nih.gov/gtr/>; accessed 11-01-2016). Currently, all FX testing is performed as LDPs: No FDA-cleared assay is available. PCR primers and Southern blot reagents are available commercially

as analyte-specific reagents (ASR) or as investigational use only. Clinical laboratories use these commercial reagents, or design primers or probes, combine them internally to develop the assay and establish performance characteristics. The ACMG has published standards and guidelines for clinical laboratories that perform this test.⁹⁸ Reference materials to standardize sizing were developed through the Genetic Testing Reference Material program⁹⁹ sponsored by the Centers for Disease Control and Prevention (CDC), the National Institute of Standards and Technology (<https://www.nist.gov/node/608501>, Accessed November 1, 2016), and the World Health Organization.¹⁰⁰ Proficiency testing through the CAP has demonstrated the excellent performance of clinical laboratories of this high-complexity LDP.¹⁰¹

Next-Generation Sequencing for Heritable Gene Panel Testing

As the genetic basis for many human diseases became apparent, sequence-based diagnostic testing was implemented as a way to provide definitive diagnoses for patients and their families. Many laboratories began offering sequence-based testing for heritable disorders in the 1990s, using a variety of mutation-scanning techniques, such as single-strand conformation polymorphism, denaturing HPLC, MLPA, and others.¹⁰² Sanger-based sequencing was the gold standard, despite being slow and expensive. The multigenic nature of some of these disorders made these sequencing approaches challenging due to the sheer number of genes and size of the sequence requiring analysis. In recent years, however, most of this testing has been converted to NGS, which offers significant advantages in terms of analytic capabilities, quality, speed, and cost.¹⁰³ The repetitive sequence reads in a single region ensure an enhanced level of quality, and NGS assays can be designed to interrogate anything from small to large gene panels, whole exomes, and beyond, depending on the clinical need being addressed. Consensus guidelines for NGS assays have been developed by multiple professional societies and address the development, validation, and quality control of these assays.^{104,105} The CAP has developed inspection checklists for laboratories performing NGS for detection of somatic mutations in cancers as well as germline mutations that cause heritable diseases. Progress has also been made in the planning and production of reference materials and proficiency samples.¹⁰⁶ Together, these practices and resources have yielded laboratory-developed assays that can be demonstrated to meet the quality levels needed for patient care.

In addition to continuous quality assessment of the “wet laboratory” procedures, ongoing national and international efforts to share data, and construct and maintain up-to-date curated databases for variant interpretation, are critical for quality care. As new mutations and variants are detected, interpretation and assessment of clinical impact, including determination of the clinical importance of variants of undetermined significance, is critical. Rapid generation of genomic data, disorganized data sharing, and a lack standardization have created challenges, and a more consistent approach to clinical sequence

interpretation is needed, along with centralized and openly accessible databases for sequence and clinical information.

In recognition of the urgent need for up-to-date variant classification resources, the ACMG and Association for Molecular Pathology released a landmark guidance document in 2015,¹⁰⁵ which has been implemented by US and international laboratories. Additional resources are outlined and include NCBI’s ClinVar database (<https://www.ncbi.nlm.nih.gov/clinvar/>), which has quickly become a valuable centralized resource for clinically classified variants, and the Clinical Genome Resource (ClinGen, www.clinicalgenome.org), which serves as a centralized site for managing genomic knowledge surrounding genes and variants. With exome and genome sequencing being increasingly implemented, guidance has been issued by the ACMGG on how to deal with the incidental identification of variants in the so-called “actionable” genes in patients tested for unrelated conditions.¹⁰⁷ Additionally, quality assessment focusing on the informatics pipeline and variant interpretation could effectively utilize sequence data sets, as has been recently outlined.¹⁰⁸

The genetics and pathology communities are increasingly embracing data sharing, which will lead to needed improvements in divergent interpretation of gene sequence variants. These interpretative tasks would be beyond the scope of an FDA approval for a kit, or the CLIA oversight of an LDP, but have a critical impact on patient care based on genomic information.

Next-generation sequencing analysis of a variety of gene panels has become routine in clinical care and has had a positive impact on the diagnosis and treatment of patients and families with complex syndromes and disorders. Examples of 3 of the most common clinical settings in which gene panels are tested for potential germline mutations are provided below, along with information on the clinical setting, potential benefits, and also challenges in utilizing these approaches.

Inherited cardiomyopathies are common disorders and include hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy, and restrictive cardiomyopathy.^{109,110} Several characteristics of all inherited cardiomyopathies provide compelling reasons for genetic testing and include (1) a substantial genetic component with detection rates currently ranging between 30% and 50%,^{111,112} (2) long presymptomatic phases with acute disease onset typically not before adolescence, and (3) a predisposition to sudden cardiac death (SCD), which can be the first presenting sign. Frequent and heartbreaking publicity following cases of sudden death of competitive athletes has brought these diseases to public attention,¹¹³ and a recent study shows that 16% of SCD cases are due to an underlying unrecognized inherited cardiomyopathy.¹¹⁴ The importance of these heritable abnormalities is underscored by the fact that nearly a third of the genes for which the ACMG recommends return of results, regardless of the test indication, are made up of these cardiomyopathy genes.

These disorders are heterogeneous, which can lead to clinical diagnostic uncertainty or error; hence, large multigene panels are particularly useful to cover the spectrum of genes

that may cause a clinical disorder. Importantly, the identification of pathogenic variants in affected individuals can inform medical management of family members and can identify those at risk early and also release negative individuals from clinical screening.¹¹¹ Genetic testing can also identify phenocopies such as Fabry disease, which can masquerade as isolated HCM. While Fabry disease is rare, disease-modifying treatment is available, and therefore, genetic testing provides essential clinical information for these patients.

Epilepsy is a common disorder of the central nervous system characterized by periodic loss of consciousness with or without convulsions associated with abnormal electrical activity in the brain. This can significantly affect the quality of life and has major psychological and socioeconomic consequences. It is estimated that there are more than 50 million people with epilepsy worldwide, and an estimated 1 in 26 people in the United States will develop epilepsy at some point in their lifetime. A significant proportion of cases show a familial distribution, and there is an increased risk of epilepsy with a family history. The prime requirements for successful management of epilepsy are a complete diagnosis and selection of an optimal treatment to benefit the patient.

Development of epilepsy may involve multiple gene abnormalities or a gene abnormality in concert with an environmental trigger. More than 100 genes have been shown to be associated with epilepsy, and a precise genetic diagnosis can help in deciding the accurate treatment and follow-up.^{115,116} Evaluation of all genes implicated in epilepsy is most efficiently accomplished using an NGS panel to provide accurate information to the physician for treatment planning. Next-generation sequencing assays are performed as LDPs under CLIA.

An example of such one gene is *SCN1A*, which causes Dravet syndrome and can be successfully treated.¹¹⁷ It is important to avoid treatment with sodium channel blockers as these can worsen seizures in Dravet syndrome. These include phenytoin (Dilantin), fosphenytoin (Cerebyx, Prodilantin), carbamazepine (Tegretol), and other medications. As ongoing research reveals new genes and mutations relevant to these diseases, it is important to classify newly found variant quickly and accurately. Open access to new information and consensus efforts to define standards for classification and reporting of variants and VUSs will be critical in ensuring that patients get the most up-to-date and complete information from genomic testing.^{104,105}

Another example is the recently discovered gene *TBCK*-related epilepsy.¹¹⁸ *TBCK*-related intellectual disability syndrome is rare with developmental delay, hypotonia, and seizures. Children with lower levels of the *TBCK* protein have slower cell mTOR signaling, which can be improved by the addition of leucine, which may provide future therapeutic options. It is important that clinical laboratories adapt their NGS panels to incorporate new targets as clinical utility is established.

Neuromuscular diseases (NMDs) refer collectively to the many disorders that affect the peripheral nervous system, either by impairing the proper development or functioning of muscles or by damaging the associated nerves or

neuromuscular junctions. Muscular dystrophies that form the majority of inherited NMDs share clinical, genetic, and pathological characteristics, including muscle degeneration and wasting, progressive muscle weakness, hypotonia, and variably elevated serum creatine kinase levels. Cardiac involvement is often present, accounting for high morbidity and mortality. There are 80 different genetically defined types of muscular dystrophies categorized based on the age of onset, the specific muscles involved, and common characteristic clinical features.¹¹⁹⁻¹²² Congenital muscular dystrophies and limb-girdle muscular dystrophies are the 2 major subgroups; these are genetically heterogeneous, with many new genes being implicated in recent years. Lack of pathognomonic signs or specific biochemical markers and the presence of high phenotypic overlap with other forms of NMDs make diagnosis difficult.

Molecular assessment is critical not only to establish a diagnosis but also to allow participation in clinical trials of therapeutic treatments that are designed for a specific set of variants or variant types. An extensive diagnostic workup involving protein studies on muscle biopsy may be used to narrow the number of single genes to be tested, but many patients never are specifically diagnosed. Comprehensive approaches to expedite molecular diagnosis now include NGS-based panel testing for sequence analysis of all disease-associated genes in a single analysis.¹²³⁻¹²⁵

Heritable Cancer Panel

Genomic testing for familial cancer syndromes has become routine over the past 2 decades. Approximately 50 heritable cancer syndromes are recognized and are causative of 5% to 10% of all cancers. Although familial breast cancer has perhaps become the most publicly known example of testing, genes for other inherited cancer syndromes may be included in NGS multigene panels. Patients who carry a germline mutation of *BRCA1* or 2 have a lifetime risk of breast cancer of 60% to 70%. The US Preventative Task Force recommends *BRCA1* and 2 testing for women who have family members with breast, ovarian, fallopian tube, or peritoneal cancer or meet other criteria.¹²⁶ Numerous studies have demonstrated the psychosocial benefits of genetic counseling and testing.^{126,127} For patients who carry a germline mutation of *BRCA1* or 2, surgical interventions may significantly reduce the risk of cancer or death. Contralateral mastectomy has been shown to reduce the risk of death in carriers by 48%.¹²⁸ Prophylactic salpingo-oophorectomy has been shown to dramatically reduce the mortality due to ovarian cancer or breast cancer in *BRCA1* mutation carriers.¹²⁹ Similarly, identification of Lynch syndrome mutations can permit surveillance leading to earlier detection and marked improvement in survival in patients developing colorectal, endometrial, or ovarian cancer.¹³⁰

Although more than 2 decades have passed since sequence-based analysis of high-penetrance cancer genes has been performed, only laboratory-developed procedures have been available. Countless patients and families have benefited from the availability of these LDPs.

Rare Disease Detection by Next-Generation Sequencing: Epidermolysis Bullosa

Epidermolysis bullosa (EB) is an inherited skin and connective tissue disease that causes skin and oral blistering with only mild trauma (http://www.niams.nih.gov/health_info/epidermolysis_bullosa/epidermolysis_bullosa_ff. Accessed November 4, 2016). The severity of the disorder depends on the layer of skin where the tissue separation occurs.¹³¹ Approximately 99% of patients with biopsy-proven EB will have mutation(s) in 1 of 18 genes known to cause the disorder (http://www.niams.nih.gov/health_info/epidermolysis_bullosa/epidermolysis_bullosa_ff. Accessed November 4, 2016). Knowledge of the specific gene can direct therapy and provide reproductive options for the family. For many years, it was possible only to sequence the suspect genes one by one, taking months to years, and only on a research basis. Recently, with the advent of NGS technology, a small number of clinical laboratories have stepped in to develop a rapid multigene NGS approach that provides answers quickly in time to make treatment decisions as well as to provide carrier and prenatal testing in at-risk families.^{131,132} As new candidate genes have been identified, NGS has been validated and offered for clinical testing.

Under CLIA, the validation data collected by the laboratory was subject to ongoing peer review, and the laboratory participates in ongoing PT to demonstrate assay quality. As the disease is rare (200 children born with EB annually),¹³³ the cost of bringing such a test through FDA for approval is prohibitive. Without an available LDP, patients and families affected by this disease would go without specific diagnoses, be unable to enroll in gene/mutation specific therapies (currently in development by at least 4 pharmaceutical companies and academic centers) (<http://www.debra.org/research-trials>. Accessed November 4, 2016), or have control over their reproductive lives.

Whole-Exome Sequencing

Whole-exome sequencing (WES) involves evaluating the coding regions of all ~20 000 human genes at once, to search for the underlying molecular cause of an undiagnosed but presumed genetic disorder. These tests are used for patients who already have undergone extensive genetic diagnostic testing and exhausted the (limited) FDA and LDP single-gene tests or in cases where it is more cost- and time-effective to start with WES. Whole-exome sequencing is used for the so-called “diagnostic odyssey” patient and has a high diagnostic yield for these patients, with 25% to 30% of studies yielding a diagnosis.^{134,135} The tests are highly complex and involve capture of the relevant DNA segments, sequencing of those segments, bioinformatics approaches to sequence alignment and identification of variants (differences between patient DNA and reference), interpretation of the identified variants, and report generation. Under CLIA and NYSDOH, the technical validation data collected by the laboratory prior to offering WES are required, and the laboratory participates in ongoing PT (through CAP and sample exchanges) to demonstrate assay quality.

As this is cutting-edge science and medical practice, the capture and sequencing technology, bioinformatics, and interpretation tools are evolving at a very fast pace. To provide the best service to patients, laboratories must frequently update, revalidate, and offer new services. Whole-exome sequencing is offered as an LDP by laboratories which have extensive experience in genetic testing. The time delay involved in bringing this test and its frequent modifications to the FDA is prohibitive. As these tests serve the rare disease community, and reimbursement is limited by the lack of pricing for a specific CPT code, the cost of bringing WES through FDA approval would be a major deterrent. Innovation would be slowed, and likely several laboratories would remove the test from test menus.

Huntington Disease

With 30 000 affected individuals in the United States,^{136,137} Huntington disease (HD) falls under the category of a rare (or orphan) disorder, and given its limited market, no commercial genetic testing platform has been developed or submitted to the FDA for review. The relatively small number of laboratories offering diagnostic or predictive (presymptomatic) testing for this disorder must therefore rely entirely on LDPs, without which patients with HD and their at-risk relatives would have no access to testing and diagnosis.

Despite the characteristic clinical features (the movement disorder [chorea] along with intellectual decline), and lack of preventive or curative treatment for HD, genetic testing is widely relied upon by HD families and their physicians. Onset of symptoms is often insidious and nonspecific, and definitive early diagnosis can only be accomplished at the DNA level.¹³² Presymptomatic testing, which is offered to the adult offspring of patients with HD (who are at 50% risk of inheriting this autosomal dominant disease), can only be accomplished using LDPs and allows crucial life-planning decisions, such as educational pursuits and career choices, marriage, and whether to have children (and if so, affording the ability to pursue prenatal diagnosis) and whether to begin planning for inevitable disability. Without this test, all of these at-risk relatives (of which there are an estimated 200 000 in the United States)¹³⁷ would lead their lives anxiously waiting for the symptoms to begin, when half of them are actually at no risk because they did not inherit the mutant gene from their affected parent.

Although there are at-risk relatives who choose not to avail themselves of the predictive test, the many who do opt to be tested credit it with freeing them of years of obsessive uncertainty. The results can afford the affected patients the opportunity to enroll in clinical trials (involving drugs or neuronal stem cells), with the aim of preventing or delaying the onset of symptoms.¹³⁸ Although these studies are still in an early phase with no outcome data yet available, they give patients some hope for the future, perhaps for themselves but also for future patients. Given the mechanism of gradual neuronal cell death in the basal ganglia, it stands to reason that the earlier such intervention is initiated—ideally in the presymptomatic stage—the higher the chances of success.

Huntington disease is one of the trinucleotide repeat disorders, caused by expansion of a tandem repeat of CAG in the first intron of the *huntingtin* (*HTT* or *IT15*) gene. In contrast to FX syndrome and some of the other trinucleotide repeat disorders, the difference between the mutated and nonmutated repeat length can be as little as a single repeat (ie, 3 nucleotides). Thus, extreme care is required in the sizing of the repeat, especially when it falls near the cutoff length of 40⁴⁰ repeats or higher which is diagnostic or predictive of HD, with 100% penetrance. Fortunately, the LDPs in current use, relying on capillary electrophoresis, are very accurate in determining repeat length, as attested by the excellent performance in CAP proficiency surveys.

Other Analytes

Busulfan

Busulfan is a bifunctional DNA alkylating agent typically given to patients as a conditioning agent prior to hematopoietic cell transplantation (HCT) for the treatment of hematologic malignancies. Therapeutic drug monitoring (TDM) is crucial for the safe and efficacious use of busulfan due to a narrow therapeutic index based on area under the curve (AUC) calculations. Too low a dose places the patient at risk of either graft failure or early relapse.^{139,140} On the other hand, too high a dose increases the risk of neurotoxicity¹⁴¹ as well as a severe and life-threatening complication termed hepatic sinusoidal obstruction syndrome (SOS).^{139,142} Hepatic SOS, previously termed hepatic veno-occlusive disease (VOD), refers to the occlusion of terminal hepatic venules and hepatic sinusoids. Severe cases of VOD can lead to hepatorenal syndrome, causing multi-organ failure, hepatic encephalopathy, and death. Venous-occlusive disease typically occurs in the context of HCT, particularly after administration of conditioning regimens prior to HCT. It is one of the most feared complications of HCT and accounts for a significant fraction of HCT-related mortality.¹⁴³ Severe cases, which account for approximately 25% to 30% of SOS, are almost always fatal.^{142,144,145}

Despite a clear need for busulfan TDM, there are currently no FDA-approved assays available for the quantitation of busulfan in blood. For this reason, various bioanalytical methods have been developed¹⁴⁶ and are currently in use by multiple laboratories. Data from a busulfan proficiency program organized by the University of Washington/Seattle Cancer Care Alliance show a total of 24 participating laboratories at the present time. Of these, 6 laboratories use gas chromatography (GC) methods, 3 use HPLC methods, and 14 use liquid chromatography–tandem mass spectrometry (MS) methods.¹⁴⁷ All of these methods are non-FDA-approved tests independently developed and validated for clinical use by their respective clinical laboratories. The use of these methods is also driven by the need for high precision and accuracy. Current criteria for acceptable laboratory performance in the analysis of busulfan is $\pm 10\%$ of the known concentrations for medium and high concentrations and within $\pm 15\%$ of the known concentration

for low concentrations.¹⁴⁷ This is due to the fact that dosing change decisions are based on AUC calculations, which depend on blood concentrations of busulfan measured from multiple timed blood draws. Multiple small analytical errors can easily add up to big differences in calculated AUC values.

Busulfan testing is currently available from reference laboratories. However, many busulfan regimens call for intravenous infusions every 6 or 24 hours for 3 to 4 days. Bone marrow transplant teams need quicker turnaround times than can be reasonably provided by sendout testing in order to be able to make dosing adjustments within this limited timespan. Guidelines have also been recently published by the American Society for Blood and Marrow Transplantation Guidelines Committee advocating for personalized busulfan dosing using busulfan TDM in certain busulfan regimens.¹⁴⁸ For these reasons, laboratory-developed methods for busulfan will continue to play key roles in the management of hematologic malignancies at cancer centers throughout the world.

Testosterone

Very sensitive measurements of serum androgens are important in adult and pediatric endocrinology and oncology. Very-low-level testosterone (Te) measurements are needed for adult women, whose values are routinely <50 ng/dL, in children, and men undergoing antiandrogen therapy whose values are usually <10 ng/dL.¹⁴⁹

The most commonly used methods for steroid analysis are FDA-approved immunoassays because they are rapid and sensitive enough for most routine applications involving healthy adult males. However, Te immunoassays lack the sensitivity requirements for chemically castrated males, women, and children. Many immunoassays also lack specificity and accuracy as immunoassays may show cross-reactivity with structurally similar compounds.¹⁵⁰⁻¹⁵² In addition, most immunoassays are not standardized against internationally recognized standards. For these reasons, a number of sensitive and specific assays using MS have been described for Te.¹⁵³⁻¹⁵⁵

The lack of sufficient accuracy and standardization of Te assays is a major concern for the clinical and public health communities.¹⁵⁶ Several years ago, the Endocrine Society, in partnership with the CDC, convened a meeting with various relevant professional societies and industrial partners to create the Partnership for the Accurate Testing of Hormones (PATH) whose mission is to improve the accuracy and standardization of a variety of steroid hormone tests.¹⁵⁶⁻¹⁵⁸ The PATH has worked with the CDC and begun to address this concern through its HoSt program, which provides laboratories with specimens spanning their analytical measurement range that have been previously analyzed using the CDC reference method.¹⁵⁵ Many assays using MS have been approved by the CDC HoST program,¹⁵⁹ however not a single FDA-approved immunoassay has met the performance requirements. Testosterone is a perfect example of an LDP that is indispensable for patient care and allows for accurate measurements to be made

on children, women, and male patients with cancer receiving antiandrogen medication.

Ethylene Glycol

Ethylene glycol is a colorless, sweet-tasting liquid commonly encountered in automobile antifreeze. Because of this widespread availability, it is also a commonly encountered toxicological agent in both accidental and self-inflicted poisonings with 6078 exposures in 2014.¹⁶⁰ Ethylene glycol poisoning classically presents with a metabolic acidosis caused by the production of toxic metabolites, primarily glycolic acid and oxalic acid. This is also often accompanied by an anion gap and osmolal gap. Untreated ethylene glycol poisoning can also progress to acute renal failure when high levels of oxalate anions combine with calcium to develop crystals in the kidneys and urinary tract.¹⁶¹

Ethylene glycol poisoning is an urgent, toxicological emergency. Once ethylene glycol is identified, the drug fomepizole is typically administered. Fomepizole inhibits alcohol dehydrogenase, the enzyme that metabolizes ethylene glycol, to slow the accumulation of toxic metabolites. Fomepizole and ethanol dramatically lengthen the half-life of ethylene glycol, and therefore, hemodialysis is often required to clear the poison.¹⁶²

Both the diagnosis and treatment of ethylene glycol poisoning are heavily dependent on laboratory measurements. No FDA-approved assays for ethylene glycol are currently available, and all testing is performed by laboratory-developed procedures. The 3 most common methods for the analysis of ethylene glycol are GC with flame ionization detector, GC with MS, and enzymatic assays.^{163,164} Gas chromatography with mass spectrometry is considered the gold standard for the analysis of ethylene glycol, as it can differentiate it from interferences that plague the other 2 methods.^{163,165} In addition to initial detection needed for diagnosis, the ethylene glycol blood concentration is used to determine when hemodialysis has cleared ethylene glycol to undetectable levels.

Thyroglobulin

Measurement of thyroglobulin (Tg) in serum has proven useful for detecting recurrence of treated differentiated thyroid carcinoma (DTC). According to the American Cancer Society, the United States has over 60 000 new thyroid cancer cases each year. The death rate is almost 2000 per year. Differentiated thyroid cancer accounts for over 90% of cases. Differentiated thyroid cancer produces Tg, making its measurement useful as a tumor marker for detecting recurrence. The NCCN and American Thyroid Association (ATA) guidelines recommend Tg testing following total thyroidectomy and radioiodine ablation treatment, including tests at baseline, 6 to 12 weeks after treatment, 6 months, 12 months, and annually thereafter. Patients free of disease have undetectable Tg.

Older competitive Tg-RIA methods are available but produce falsely high Tg results in the presence of Tg autoantibodies (Tg-Ab).¹⁶⁶ Newer FDA-cleared Tg assays are

immunometric immunoassays (Tg-IA) and can detect Tg at concentrations down to approximately 0.1 ng/mL. Generally, Tg is captured with a solid-phase antibody, then quantitated using a detection anti-Tg reagent. The signal is directly proportional to the amount of Tg. The assay design is susceptible to interference from endogenous anti-Tg-Ab.¹⁶⁶ The intended use of these Tg-IA are Tg measurement in Tg-Ab-negative (Tg-Ab⁻) patients. The FDA requires Tg-Ab testing whenever Tg is measured using these cleared methods.

Depending on the method used, up to 36% of treated patients with DTC are Tg-Ab positive (Tg-Ab⁺). Thus, many of these patients have falsely low or even falsely negative Tg results when measured by IA. Four Tg-Ab assays are in common use and are not harmonized, detecting Tg-Ab in widely divergent numbers of treated patients with DTC.¹⁶⁷ In addition, the degree of Tg interference cannot be predicted from the magnitude of the Tg-Ab result. Thus, up to 20% of patients with recurrence are missed by Tg-IA testing.

To circumvent the Tg-Ab interference, Hoofnagle and Wener developed and validated a MS Tg method (Tg-MS) using tryptic digestion and immunocapture of Tg-specific peptides followed by MS focused on those peptides.¹⁶⁷ They demonstrated the Tg-MS method accurately measures Tg in the presence of Tg-Ab.¹⁶² The tryptic digestion destroys the Tg-Ab, eliminating their interference with the assay. Four national reference laboratories have adopted versions of this method, and harmonization efforts are underway. A recent clinical outcome study compared Tg-IA, Tg-RIA, and Tg-MS in both Tg-Ab⁻ and Tg-Ab⁺ patients. As predicted from the assay designs, all methods were equivalent for Tg-Ab⁻ cases, but the Tg-MS was more accurate for Tg-Ab⁺ cases. Tg-IA methods had more false negatives and Tg-RIA had more false positives.¹⁶⁸

Although Tg-MS has not yet been incorporated into the current guidelines, the ATA guideline mentions it as a promising new technique. Without Tg-MS, thyroid cancer recurrence in Tg-Ab⁺ patients can be missed, thus delaying follow-up and creating patient harm.

Antimicrobial Susceptibility

Antimicrobial susceptibility testing, used to determine whether antibiotic treatment will be successful, is an essential component of the microbiology culture report. Emerging resistance among pathogenic bacteria and new antimicrobial agents require frequent updates to both testing methods and interpretation of the results. Most laboratories use automated instruments to perform minimal inhibitory concentration (MIC) testing to determine whether a patient's isolated bacteria are susceptible, susceptible dose dependent, intermediate, or resistant to a panel of antibiotics. These interpretations are based on FDA breakpoint criteria published at the time of drug approval and periodically updated to respond to the appearance of new resistance mechanisms.¹⁶⁹ The FDA also clears automated instrumentation used to determine MIC values (via the 510k process).¹⁷⁰ Although the MIC testing process may not be

changed, new breakpoints added to the instrument software require a revised 510k application. Because the FDA does not have the authority to require manufacturers to submit data for revised breakpoints within a specified time frame, manufacturers may elect to use outdated breakpoints rather than face the expense of a 510K resubmission. A recent example was the 3-year delay between the release of updated breakpoints for diagnosing carbapenem-resistant *Enterobacteriaceae* in 2010 and the ability to use these breakpoints in the clinical laboratory. This delay was used to calculate the potential for additional carriers of multidrug-resistant *Enterobacteriaceae* in Southern California health-care systems. As many as 1821 additional carriers of MDRO *Enterobacteriaceae* were estimated to have occurred in Orange County, California because of this delay.¹⁷¹ The disastrous spread of MDRO *Enterobacteriaceae* can be mitigated by laboratories validating testing methods that enable use of updated antimicrobial breakpoint interpretations before FDA-cleared testing is available. Specifically, modifying a manufacturer's instructions, including interpreting MIC results using a revised breakpoint other than that listed in the product insert, is a change that renders the procedure an LDP. Without the option of using an LDP, one is left reporting outdated interpretations that miss resistant strains leading to unacceptable patient care.

Conclusion

As illustrated, LDPs are an integral part of the spectrum of tests and procedures performed by clinical laboratories which fulfill a critical need for patient care, particularly in rapidly evolving areas such as testing for personalized medicine. Laboratory testing should be consistent with national/international consensus treatment guidelines, which may require development of procedures earlier or for new clinical purposes not fulfilled by FDA-approved kits. In such cases where clinical testing needs exist beyond the original FDA purpose, laboratories must be able to perform additional validation of new sample types or develop additional assays to include new mutations or analytes that are needed. As illustrated by these case examples, laboratories and professional organizations often work together to broadly compare and optimize assay performance, creating consensus standards that raise the quality of testing overall. In contrast, the current structure for FDA approval requires review of assay kits individually, or in comparison with the predicate method, rather than assessing and improving performance across the spectrum of assay options available.

The science of laboratory medicine has advanced dramatically in the almost 3 decades since CLIA was enacted, and updates and expansions to CLIA regulations could be useful. Additional resources, such as reference materials, and consensus practice guidelines would extend the quality framework that all laboratories and manufacturers utilize. For example, consensus guidelines that include such details as the target for percent allele frequency detectable, requirements for percent tumor cell content, what mutations and variants should be

included, and sample types to be tested would be useful as a guide for assay validation as well as in standardization of the practice. Professional expert groups are already generating assay and practice guidelines.^{69,73,172-175} Ideally, clinical laboratories and kit manufacturers would utilize appropriate reference materials to help standardize the results obtained for any particular analyte regardless of technology platform or laboratory setting.

To address the needs for reference materials to facilitate assay result standardization, a multistakeholder initiative, the Diagnostic Quality Assurance Pilot, has been launched to design, develop, and evaluate traceable reference sample materials (referred to as reference materials) to better provide molecular pathology laboratories with the means to demonstrate equivalent performance of LDPs and companion diagnostic IVDs for targeted cancer therapy. This Quality Pilot emerged from the Sustainable Predictive Oncology Therapeutics and Diagnostics working group, launched in 2013 by Tapestry Networks (Waltham, Massachusetts), which was composed of diverse stakeholders (oncologists, pathologists, patient advocates, third party payers, and regulators) for the purpose of designing a quality pilot to advance these goals (<http://www.tapestrynetworks.com/initiatives/healthcare/oncology-therapeutics-and-diagnostics/diagnostic-quality-assurance-pilot.cfm>. Accessed April 27, 2017).¹⁷⁶ The Tapestry pilot proposes that laboratories would be allowed to utilize assays that best serve the needs of their patients based upon performance, quality, clinical needs, and the test menus and volumes of that particular laboratory. It is not critical that laboratories all use the identical assay or test platform, provided that all are able to get the correct answer.

To close, the overarching goal is the efficacy and safety of our clinical laboratory tests and procedures for patients. Pathologists and laboratory professionals need the best and most up-to-date tools to do their jobs and optimize patient care. Some of these will be FDA approved or cleared kits, and others will be laboratory-developed procedures performed under CLIA; both have their place. Laboratories have a long history of success performing LDPs, as illustrated by these case studies.

As much as possible, these capabilities need to be performed on-site to insure that the results can be integrated with other clinical and laboratory findings, interpreted as a whole and completed in a timely fashion. Also important is the hands-on training of the next generation of physicians, for whom, we hope, maximal use of genomic and other laboratory information will be a way of life as they treat human disease. That is the promise of personalized medicine!

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